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(54) Title: COMPOSITIONS AND METHODS FOR TARGETED DELIVERY OF EFFECTOR MOLECULES

(57) Abstract

Targeting and effector molecules are conjugated by covalent attachment of the targeting molecule to the effector molecule by a combination of imine formation and thioalkylation. The conjugates are used in therapeutic or diagnostic applications to target the effector molecule to a desired cell or tissue. The immunoconjugates are particularly useful in the treatment of tumors, where, for example, antibody-doxorubicin conjugates are effective against doxorubicin resistant tumor cells.

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COMPOSITIONS AND METHODS FOR TARGETED DELIVERY OF EFFECTOR MOLECULES

Background of the Invention

The present invention relates generally to compositions and methods for targeting effector molecules to specific tissues in the body. More specifically, the present 10 invention provides targeting molecule-effector moiety conjugates in which covalent attachment of the targeting molecule to the effector moiety is made by a combination of imine formation and thioalkylation.

The therapeutic usefulness of many drugs, particularly those used in the treatment of cancer, is limited 15 by toxicity against normal host cells. By their very nature, antineoplastic agents are toxic to many mammalian cells. Thus, the dose of an antineoplastic agent that can be administered to a patient is limited by the adverse effects 20 suffered by normal host tissues. Unfortunately, in many instances doses that may be tolerated by most patients are not sufficient to eradicate the neoplastic disease. Because of the low therapeutic index of such drugs, efforts have been made to direct the therapeutic compounds to specific targeted 25 cells or tissues, thereby providing a higher drug concentration at sites where the therapeutic effect is desired. A variety of methods have been attempted to achieve such targeting.

For example, the use of antibodies for the targeted 30 delivery of therapeutic agents was first suggested by Ehrlich in the early years of this century (see, Ehrlich, P: In *Collected Studies on Immunity*, Vol. 2, pp. 442-447, Wiley, New York, 1906). Nevertheless, it was over 50 years before an attempt was made. In this effort, methotrexate was diazo-linked to an antibody raised against a mouse leukemia (see, 35

Mathe *et al.*, *C. R. Acad. Sci. (D)*, Paris **246**:1626 (1958)). In the years since that effort there has been a dramatic increase in research directed to the targeted delivery of compounds using antibody conjugates.

5 More recently, monoclonal antibodies have been investigated as a way of achieving site-specific delivery and possibly improving the therapeutic index of chemotherapeutic drugs (see, Hellstrom *et al.*, *Controlled Drug Delivery, Fundamentals and Applications*, 2nd ed. New York, Marcel Decker, pp. 623-653 (1987), incorporated herein by reference). Conjugation of drugs to antibodies may also lower uptake in normal tissues, thereby decreasing blood clearance and metabolic degradation and improving bioavailability at the tumor site. In spite of these potential advantages, poor 10 tumor penetration, low antigen expression and antigenic heterogeneity may limit the number of antibody-targeted drug molecules that can reach tumor cells. These problems have been addressed either by using highly potent drugs for conjugation, or by achieving the maximum possible loading of 15 conventional chemotherapeutic drug molecules on an antibody molecule while preserving the antibody's immunoreactivity (see, Reisfeld, *et al.*, *Therapeutic Monoclonal Antibodies*, Borrebaeck and Larrick (eds.), New York, Stockton Press, p. 20 57-73 (1990), incorporated herein by reference).

25 A variety of methods have been used to couple a therapeutic agent to a targeting molecule such as an antibody. One method for the direct coupling of an antibody to a therapeutic agent involves modification of the antibody's amino functions with a crosslinking agent such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). See, 30 Heath, *et al.*, *Proc. Natl. Acad. Sci. USA* **80**:1377-1381 (1983). However, such modifications are not selective and may damage the antigen binding site or may affect the antibody in such a manner that the antigen binding sites are not available for 35 binding.

Alternatively, antibodies may be coupled to therapeutic agents following oxidation of the carbohydrate functions located in the CH₂ domain of the Fc fragment. These

procedures allow selective modification of the antibody such that the antigen binding sites are not damaged and are always directed outward from the therapeutic agent (see, Domen, et al., *J. Chromatography* 510:293 (1990) in which the therapeutic agent is associated with a liposome). The most common procedure of this sort involves coupling the oxidized antibody to a therapeutic agent containing amino functions by reductive amination using sodium cyanoborohydride. Aggregation may result from such systems due to reductive amination occurring with the amino functions of a second antibody.

One class of therapeutic agents which have been investigated for targeted delivery are the anthracyclines, which include, for example, doxorubicin. These are among the most potent anticancer agents known, but their clinical application is limited by cardiotoxicity. Several investigators have tried to overcome this limitation by attaching the compounds to a carrier system such as an anti-tumor monoclonal antibody to deliver the drug to tumor sites. Studies in preclinical models demonstrated that immunoconjugates of doxorubicin immunoconjugates can kill tumor cells more effectively than equivalent doses of free drug (see, e.g., Dillman, et al., *Cancer Res.*, 46:4886-4891 (1986); Yang, et al., *Proc. Natl. Acad. Sci. USA* 85:1189-1193 (1988); Aboud-Pirak, et al., *Proc. Natl. Acad. Sci. USA* 86:3778-3781 (1989); Braslawsky, et al., *Cancer Res.* 50:6608-6614 (1990); Shih, et al., *Cancer Res.* 51:4192-4198 (1991); Trail, et al., *Cancer Res.* 52:5693-5700 (1992); and Trail, et al., *Science* 261:212-215 (1993) the disclosures of all being incorporated herein by reference).

Despite these efforts, a number of the doxorubicin immunoconjugates suffer from 1) hydrolytically sensitive linking groups which result in premature release of the drug from the targeting moiety, 2) limitations on the amount of drug which can be attached to the targeting molecule, or 3) reduced cytotoxicity. The latter limitation has been attributed to increased stability of the drug-antibody linking group (see, Mueller et al., *Bioconjugate Chem.* 1:325-330 (1990)).

What are needed in the art are alternative methods and compositions for targeting therapeutic agents to specific tissues. These new methods should provide increased ratios of the therapeutic agent to the targeting group, and possess a linkage which is stable in serum, yet allows a high accumulation of the therapeutic agent at the target site without the risks associated with direct local administration. Quite surprisingly, the present invention fulfills these and other related needs.

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Summary Of The Invention

The present invention provides compositions and methods for selective delivery of therapeutic and diagnostic agents (effector molecules) to a target site or tissue in a host. The compositions are conjugates of effector molecules and targeting molecules, in which the targeting molecule is directed to a binding site on the target cell, tissue, etc. The conjugate is formed by covalent attachment of the targeting molecule to an imine functionality which is present in, or generated in an effector moiety. In some embodiments of the invention, the targeting molecule is covalently attached to the effector moiety (or derivatized moiety) at two or more positions. In other embodiments, the covalent attachment is at a single site.

The methods generally comprise administering the targeting molecule-effector moiety conjugates to the target site, tissue or host.

Brief Description Of The Drawings

Fig. 1 is a schematic illustration of the preparation of a doxorubicin-protein conjugate of the present invention.

Fig. 2 is a graph which illustrates the cytotoxicity of monoclonal antibody 425-doxorubicin conjugate, monoclonal antibody 425 and doxorubicin.

Fig. 3 is a graph which illustrates the antitumor activity of monoclonal antibody 425, doxorubicin and monoclonal antibody 425-doxorubicin conjugate evaluated with subcutaneous M24 met melanoma xenografts.

5 Fig. 4 is a graph which shows the survival of mice treated with free doxorubicin or monoclonal antibody doxorubicin conjugates in the melanoma metastasis model.

Detailed Description Of The Specific Embodiments

In one aspect, the present invention provides novel
10 targeting molecule-drug conjugates which have the formula:



In this formula, the symbol D¹-N represents a condensation product of an effector moiety such as a therapeutic or imaging agent having a free amino group. The symbol Y¹-P¹-Y² represents a condensation product of a targeting molecule of the conjugates, P¹, having sidechain functionalities, Y¹ and Y² which are independently -SH or -NH₂. The symbol X represents a variety of linking groups including -O-, -S-, -CH₂-, and -CH(R)-. The groups R, R¹ and R² are each independently H, lower alkyl, lower alkoxy, halogen, lower alkylthio, lower acyloxy, cyano, or nitro.
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The targeting molecule-effector moiety conjugates of formula I are represented as having a six-member ring in a "chair" conformation. However, this conformation and the axial orientation of the substituents is presented for clarity and is not meant to be limiting. Thus, no particular conformation or stereochemistry of the substituents is intended.
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As noted above, the symbol X represents a variety of linking groups including -O-, -S-, -CH₂-, or -CH(R)- in which
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R is H, lower alkyl, lower alkoxy, halogen, lower alkylthio, lower acyloxy, cyano, or nitro. Preferably, X is -O-, -CH₂-, or -CH(R)- in which R is lower alkoxy. More preferably, X is -O-, -CH₂- or -CH(OCH₃)-. The term "alkyl" refers to a saturated hydrocarbon radical of from one to twenty carbon atoms which may be straight-chain or branched-chain (for example, ethyl, isopropyl, t-amyl, or 2,5-dimethylhexyl). This definition applies both when the term is used alone and when it is used as part of a compound term, such as "alkoxy," "alkylthio" and similar terms. The term "lower alkyl" refers to those alkyl groups containing 1 to 5 carbon atoms. All numerical ranges in this specification and claims are intended to be inclusive of their upper and lower limits. For the substituents listed above, the term "acyloxy" refers to an acyl group which is linked to the remainder of the molecule through an oxygen atom. Thus "acyloxy" can be viewed as the radical which remains upon removal of the acidic hydrogen from a carboxylic acid. Examples of acyloxy substituents include, for example, acetoxy, propionyloxy, pivaloyloxy and hexanoyloxy.

The groups R¹ and R², like R, can be H, lower alkyl, lower alkoxy, halogen, lower alkylthio, lower acyloxy, cyano, or nitro, wherein the groups have the meaning provided above. Preferably, R¹ and R² are each independently H or cyano.

The effector moiety portion of the targeting molecule-effector moiety conjugates can be any of a wide variety of compounds which contain a primary amine functionality that can participate in imine formation with an aldehyde. While the invention will be described for compounds containing a primary amine functionality, one of skill in the art will understand that other compounds having other suitable functionalities can be modified to derivatized compounds having primary amine groups using conditions known to those of skill in the art. For example, compounds having an accessible hydroxyl substituent can be acylated with an amino acid such as glycine to provide the compound with a primary amine group. Similarly, compounds having thiol substituents can be alkylated with, for example, 2-bromoethylamine. Still other

compounds having carboxylic acid groups can be esterified with, for example, ethanolamine in which the amine group is initially protected with a suitable protecting group. Removal of the protecting group will provide a primary amino group.

5 Any number of similar modifications are contemplated as within the scope of the present invention. These modifications are known to those of skill in the art and can be found in March, *Advanced Organic Chemistry*, 4th Ed. Wiley-Interscience, New York, NY, p. 1167-1171 (1992), incorporated herein by reference.

10 In another aspect, the present invention provides targeting molecule-effector moiety conjugates having the formula:



15 In this formula, the letters D¹-NH represent a condensation product of an effector moiety having a free amino group. The symbol Y³-P¹ represents a condensation product of a targeting molecule P¹ having a sidechain functionality, Y³, which is -SH or -NH₂. The symbol, R³, represents an alkyl group, an alkoxy group, an alkylthio group, a halogen, a carboxylic ester group and a carboxylic acid group. The effector moieties and targeting molecules which are useful in this aspect of the invention are the same as those useful for the targeting molecule-effector moiety conjugates of formula I.

20 The targeting molecule-effector moiety conjugates of the present invention which are embodied in formulae I and II can be prepared by similar procedures. Without intending to be bound by any particular mechanism of formation, Fig. 1 depicts one method of forming the conjugates of formula I (in which doxorubicin is the effector moiety). With reference to Fig. 1, an effector moiety or imaging agent having at least one primary amine group is derivatized with a dialdehyde to form a first intermediate iminium ion. The iminium ion is

trapped by the nucleophilic addition of a first thiol functionality present in a protein or other targeting molecule. In subsequent steps, the remaining aldehyde group which was initially present in the dialdehyde, cyclizes onto 5 the nitrogen atom from the initial amine group to form a second iminium ion, which is in turn trapped by a second thiol functionality present in the protein or targeting molecule. Thus, for the preparation of the targeting molecule-effector 10 moiety conjugates of formula I, a suitable effector moiety (one which contains a primary amine or has been modified to contain a primary amine) is reacted with a dialdehyde to produce an intermediate. The intermediate is then treated with a targeting molecule such as a protein having the appropriate side chain groups. Preferably, the side chain 15 groups are reduced disulfide linkages. Reduction of disulfide linkages in proteins can be carried out by means known to those of skill in the art. Typically, the disulfide linkages are reduced by treating the protein with such reagents as, for example, dithiothreitol (DTT), mercaptoethanol or cysteine. 20 Reaction of the targeting molecule with the effector moiety intermediate takes place under suitable conditions. Such conditions include typically an aqueous solvent or a mixture of an organic and aqueous reaction medium. Additionally, the reaction is preferably conducted at temperatures of from 0 °C 25 to about 45 °C, more preferably between about 20 °C and 35 °C in an atmosphere of nitrogen, argon, or air. After a sufficient period of time, the resulting conjugate is formed in which the targeting molecule is attached to the effector moiety through one, two or more covalent linkages. One of skill in the art 30 will understand that for some targeting molecules, reduction of disulfide linkages and subsequent reaction of the reduced targeting molecule with the effector moiety intermediate will result in targeting molecules having multiple attached drugs. Following covalent attachment of the targeting molecule to the 35 drug, the conjugates are purified by, for example, thin or thick layer chromatography, membrane filtration, gel filtration, or ion exchange chromatography, etc.

A number of dialdehydes are commercially available and can be used to synthesize the targeting molecule-effector moiety conjugates of formula I. In particular, a dialdehyde such as glutaraldehyde will provide a protein-effector moiety conjugate in which X is $-\text{CH}_2-$ and R¹ and R² are both hydrogen.

5 Alternatively, the dialdehyde can be prepared *in situ* and reacted with a compound having a primary amine functionality. For example, treatment of 3-methoxy-1,2-cyclopentanediol with sodium periodate results in the formation of 3-

10 methoxyglutaraldehyde which can be reacted directly with an appropriate compound to produce a derivatized or intermediate compound which can be subsequently treated with an antibody or a reduced antibody. Still other dialdehydes are available by procedures known to those of skill in the art. For example,

15 2,2'-oxydiacetaldehyde ($\text{OHC}-\text{CH}_2\text{OCH}_2-\text{CHO}$) can be formed by acid hydrolysis of 2,2'-oxydiacetaldehyde bis(diethyl acetal) by the method of Field, et al. Belgian Pat. No. 655,436 or by cleavage of 1,4-anhydroerythritol by the method of Barry, et al. *Carbohydrate Research*, 7:299 (1968) and Greenberg, et al. *Carbohydrate Research*, 35:195 (1974).

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The targeting molecule-effector moiety conjugates of the present invention which are embodied in formula II can be similarly prepared by substituting a monoaldehyde for a dialdehyde. One of skill in the art will recognize that a wide variety of monoaldehydes are suitable for the preparation of the conjugates. Aliphatic aldehydes which do not provide steric hindrance to the subsequent attachment of a targeting molecule such as a protein are preferred.

25 Thus, in another aspect, the present invention provides methods of preparing targeting molecule-effector moiety conjugates which are useful for the targeted delivery of effector molecules. These methods comprise,

- (a) treating an effector moiety having a primary amine functionality with a dialdehyde of formula III:

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- to produce a derivatized compound; and
- (b) contacting a targeting molecule with the derivatized effector moiety under conditions sufficient to produce a targeting molecule-effector moiety conjugate wherein the targeting molecule is covalently attached to the effector moiety through one, two or more linkages.

In this aspect of the present invention, the drugs can be any of the compounds provided for the conjugates of formula I, including those compounds in which a primary amine functionality is introduced by modification of the compound's existing functionality. The reaction of the compounds with a dialdehyde of formula III can be carried out under conditions which are well known to those in the art. Typical conditions use a solvent which is substantially aqueous, but may include portions of organic solvents such as DMSO or acetonitrile. Ambient temperatures are also preferred. The dialdehydes which are used in step (a) are represented by formula III, in which X represents a variety of linking groups including -O-, -S-, -CH₂-, and -CH(R)-. The groups R, R¹ and R² are each independently H, lower alkyl, lower alkoxy, halogen, lower alkylthio, lower acyloxy, cyano, or nitro. Particularly preferred dialdehydes are glutaraldehyde, 2,2'-oxydiacetaldehyde and 3-methoxyglutaraldehyde.

The targeting molecule, e.g., a protein, carbohydrate or oligonucleotide, is contacted with the derivatized compound to produce the conjugate in which the targeting molecule is attached to the compound through two or more covalent sites of attachment. A particularly preferred targeting molecule is a protein such as a reduced or modified antibody having thiol groups which are available for covalent attachment to the derivatized compound. Reducing at least one disulfide linkage in a targeting molecule, e.g., an antibody can be carried out by any of the techniques which are known to those of skill in the art. Briefly, reduction of the targeting molecule can be carried out by treatment of the molecule with dithiothreitol or mercaptoethanol followed by chromatography to remove excess reagent and by-products. Specific conditions for the

reduction are also well known to those of skill in the art. In this manner, disulfide linkages in an antibody or other targeting molecule are reduced to provide a targeting molecule having pairs of thiol groups which are in close proximity and which can be attached to a suitably derivatized compound. Alternatively, the targeting molecule can be modified with reagents such as iminothiolane which reacts with the amino group of lysine residues to generate a thiol group. Contact of a targeting molecule such as an antibody in its native form, a fragment, or a reduced or modified version of the protein with the derivatized compound is carried out under conditions known to those of skill in the art. In particular, contact is typically carried out in a substantially aqueous medium or a polar organic solvent, for example DMF.

Additionally, the contact is performed at temperatures of from about -10 °C to about 40 °C.

The "effector moiety" portion of the targeting molecule-effector moiety conjugate, D¹-N, can be any chemical species for which targeted delivery is desired and which contains a primary amine functionality or which contains a functionality that can be modified to provide a primary amine group. Examples of such effector moiety moieties include but are not limited to therapeutic compounds such as, e.g., nucleic acids such as antisense oligonucleotides (as described in, e.g., WO 93/09813 and WO 93/01286, both incorporated herein by reference) and ribozymes (e.g., U.S. Patent Nos. 4,987,071, 5,254,678, and WO 94/26877, each incorporated herein by reference), proteins, peptides, oncolytics, anti-infectives, anti-inflammatory agents, cardiovascular agents, anxiolytics, psychotropics, ionotropes, toxins such as gelonin and inhibitors of eucaryotic protein synthesis, and the like.

Thus, among the therapeutic agents which contain an available amino group or a functionality that can be converted to an available amino group include a wide variety of antibacterial and antifungal compounds such as penicillins, cephalosporins, fluoroquinolones, sulfonamides, chloramphenicol, aminoglycosides, rifampin, metronidazole, polymyxins, erythromycin, tetracyclines, amphotericin B,

nystatin, vancomycin, acyclovir, ribavirin and zidovudine; local anesthetics such as dibucaine and chlorpromazine; antihypertensive agents such as clonidine and hydralazine; antidepressants such as imipramine, amitriptyline and doxepim; 5 antiarrhythmic agents such as quinidine and disopyramide; antihistamines such as diphenhydramine, chlorpheniramine and promethazine; beta-adrenergic blockers such as propranolol, timolol and labetolol; biologics such as interleukin-2, beta-interferon and growth hormone release inhibiting factors, such 10 as somatostatin; and antineoplastic agents.

In preferred embodiments, D¹-N represents the condensation product of an antineoplastic agent. More preferred are those embodiments in which D¹-N represents the condensation product of antineoplastic agents such as 15 doxorubicin, bleomycin, vinblastine, vincristine, etoposide and etoposide phosphate, paclitaxel, teniposide, daunomycin, melphalan, cyclophosphamide, chlorambucil, cisplatin, carboplatin, methotrexate, podophyllotoxin, verrucarin A, anguidine, mechlorethamine, leucovorin, cytosine or adenine 20 arabinosides, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine, 5-ido-5'-amino-2',5-dideoxyuridine, 5'-amino-5'-deoxythymidine, mitomycin, 1-amantanamine, rimantadine, tamoxifen, etc.

Alternatively, the drug portion of the conjugate may 25 be an encapsulation system, such as a liposome or micelle that contains a therapeutic composition such as a drug, a nucleic acid (e.g., an antisense nucleic acid), or another therapeutic agent that is preferably shielded from direct exposure to the circulatory system. Means of preparing liposomes attached to 30 antibodies are well known to those of skill in the art. See, for example, U.S. Patent No. 4,957,735, Connor et al., *Pharm. Ther.*, 28: 341-365 (1985), incorporated herein by reference.

Imaging agents are also useful in the present 35 invention as the effector moiety portion of the targeting molecule-effector moiety conjugates. As above, the imaging agents will contain either a primary amine functionality or a functionality which can be readily converted to a primary amine. Imaging agents suitable for use in this invention

include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful agents in the present invention include magnetic beads (e.g. DynabeadsTM), 5 fluorescent dyes (e.g., fluorescein isothiocyanate, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ³H, ¹²⁵I, ³⁵S, ¹⁴C, or ³²P), enzymes (e.g., horseradish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as 10 colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Means of detecting or imaging the conjugates containing these agents are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation 15 counters, fluorescent markers may be detected using a photodetector to detect emitted illumination. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels 20 are detected by simply visualizing the colored label.

The targeting portion of the conjugates, P¹, can be any number of molecules which specifically recognize or are recognized by a desired target site. More particularly, suitable targeting molecules for use in the conjugates of the 25 present invention can be any protein, polypeptide, polyaminoacid, glycoprotein, carbohydrate, antibody or antibody fragment, or nucleic acid, which is reactive with a specific target population desired to be treated or imaged, such as a cell, e.g., a tumor cell, or which is a ligand that binds specifically to a receptor associated with the cell 30 surface of a target cell population, e.g., a tumor-associated antigen.

For example, proteins suitable for use in the present invention include ligands (targeting molecule), capable of reacting with or otherwise recognizing or binding to a target receptor on or in a target cell, tissue or biological fluid, include, but are not limited to, antibodies, growth factors, lymphokines, cytokines, and hormones. These include specific 35

proteins such as epidermal growth factor (EGF), IL-2, IL-4, IL-6, IGF1, CD4, gastrin, gastrin-releasing peptide, TGF- α , TGF- β , transferrin, platelet-derived growth factor, vaccinia growth factor, insulin and insulin-like growth factors I and II, and the like which specifically bind desired target cells.

Thus, the targeting molecule can be an antibody which has been generated to an antigen associated with a particular disease state and which is reactive with a specific target cell population desired to be inhibited, eliminated, stimulated or imaged. Preferably, the antibody portion of the conjugate is any antibody which binds specifically to a tumor-associated antigen. Examples of such antibodies include, but are not limited to, those which specifically bind to antigens found on carcinomas, melanomas, lymphomas and bone and soft tissue sarcomas as well as other tumors. Antibodies that remain bound to the cell surface for extended periods or that are internalized can be used, and internalization is not required. As used herein, the term "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The basic immunoglobulin (antibody) structural unit a tetramer composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies may exist as intact immunoglobulins, or modified in a variety of forms including, for example, modified or altered antibodies having an Fv fragment containing only the light and heavy chain variable regions, a Fab or Fab'₂ fragment containing the variable regions and parts of the constant regions, a single-chain antibody (US Patent No. 4,946,778 to Ladner; Bird et al., *Science* 242: 424-426 (1988); Huston et al., *Proc. Natl. Acad. Sci. USA* 85: 5879-5883 (1988)), and the like. The antibody may be of animal (especially mouse or rat) or human origin or may be

chimeric (US Patent No. 4,816,567 to Cabilly; Morrison et al., *Proc Natl. Acad. Sci. USA* 81, 6851-6855 (1984), and Oi, *BioTechniques* 4(3):214-221 (1986)) or humanized (Jones et al., *Nature* 321, 522-525 (1986), and published UK patent application #8707252).

Additionally, the antibodies can be polyclonal or monoclonal. Methods of producing antibodies suitable for use in the present invention are well known to those skilled in the art and can be found described in such publications as Harlow & Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, (1988). Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art (see, for example, Kohler and Milstein, *Eur. J. Immunol.* 6:511-519 (1976), incorporated herein by reference). Examples of such antibodies include, but are not limited to, antibodies that bind to tumor-associated antigens such as antigens found on carcinomas, melanomas, lymphomas, bone or soft tissue sarcomas, as well as other tumors. Examples of these monoclonal antibodies include those which target the B72 antigen (U.S. Patent Nos. 4,522,918 and 4,612,282), melanoma associated antigens (U.S. Patent Nos. 5,262,177, 5,141,742, 5,055,559), CA 125 antigen (U.S. Patent No. 5,059,680), sarcoma associated antigens (U.S. Patent No. 5,059,523), and other tumor associated antigens (e.g., U.S. Patent Nos. 5,091,177, 4,939,083, 4,708,862, 4,737,579, 4,958,009, 4,753,894) (each of the foregoing patents being incorporated herein by reference). Other examples of antibodies are those that bind to virus- or other pathogen-associated antigens, and antibodies that bind to abnormal cell surface antigens.

Additionally, the antibody must possess side chain functionality, such as amino groups and thiol groups, which are capable of attachment to a suitable effector moiety. For example, side chain functionality such as -SH and -NH₂ upon covalent attachment to an effector moiety, become Y¹ and Y² (each of which is either -NH- or -S-). In some embodiments, disulfide linkages are reduced to form two reactive thiols (as Y¹ and Y²). In still other embodiments, thiol groups can be introduced into an antibody or other protein using such

reagents as iminothiolane (see, Traut, et al., *Biochemistry* 12:3266 (1973) and Jue, et al., *Biochemistry* 17:5399 (1978), the disclosures of which are incorporated herein by reference).

5 The present invention also encompasses pharmaceutical compositions, combinations and methods for treating diseases. The diseases which can be treated by the compositions of the present invention include bacterial, viral and fungal infections, cancers and other tumors, and autoimmune diseases.

10 The compositions, combinations and methods of this invention are useful in treating any mammal, including, for example, humans, cows, dogs, cats, pigs, goats, mice, sheep, rabbits and horses.

15 The pharmaceutical compositions of the present invention comprise the protein-effector moiety conjugates and conventional pharmaceutically acceptable carriers known in the art such as serum proteins (*i.e.*, human serum albumin). Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. These compositions may be sterilized by conventional, well known sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and used directly. The compositions may also contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

20 The concentration of conjugates in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

25 Additionally, the pharmaceutical compositions of the invention comprising the conjugates may be in a variety of

dosage forms which include, but are not limited to, solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, suppositories, polymeric microcapsules or microvesicles, liposomes, and
5 injectable or infusible solutions. The preferred form depends upon the mode of administration and the particular application.

In addition to the above-noted pharmaceutical compositions, the present invention also provides combinations comprising two or more different conjugates of the present invention and methods for treating tumors wherein a mammalian host is treated in a pharmaceutically acceptable manner with a pharmaceutically acceptable amount of the conjugate or conjugates. Thus, the present invention also provides methods
10 for the administration, either simultaneously or sequentially of at least two different conjugates, *i.e.*, bearing different effector moieties or different proteins, for use in methods of combination chemotherapy. For example, an embodiment of this invention involves the use of a number of anthracycline-
15 conjugates wherein the specificity of the protein component of the conjugate varies (for example, multiple antibodies are used which have specificity for different antigens or to different sites or epitopes on the same antigen). Additionally, the anthracycline component of these conjugates
20 may be the same or may vary.

The use of multiple conjugates will find application in the treatment of a tumor where the amounts of the various antigens on the surface of the tumor is unknown or the tumor cell population is heterogeneous in antigen expression and one wants to be certain that a sufficient amount of drug is
25 targeted to all tumor cells at the tumor site. Alternatively, multiple conjugates can be used in which only the drug component of the conjugate varies. For example, a particular antibody can be linked to doxorubicin to form one conjugate and can be linked to daunomycin to form a second conjugate.
30 Both conjugates can then be administered to the host to be treated and will localize, due to antibody specificity, at the site of the selected cell population. Both drugs can then be
35

released at that site. This latter embodiment will be particularly useful for those cell populations in which drug resistance has developed or is developing.

As noted above, the present invention also provides methods for the treatment of diseases, tumors or other tissue malignancies in which a therapeutically effective amount of a targeting molecule-effector moiety conjugate is administered to a patient. The most effective mode of administration and dosage regimen for the conjugate compositions of this invention depends upon the severity and course of the disease or targeted tissue, the patient's health and response to treatment and the judgement of the treating physician. Accordingly, the dosages of the conjugates and any accompanying compounds should be titrated to the individual patient. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. Nevertheless, an effective dose of the doxorubicin-conjugate of this invention may be in the range of from about 1 to about 100 mg/m² doxorubicin, or from about 500 to about 5000 mg/m² antibody.

The present invention also provides conjugates in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the various elements of the kit. For example, one element of a kit can be a vial containing a pharmaceutically acceptable composition comprising an imaging agent conjugated as described herein to a tumor targeting molecule. The conjugate is then administered to a patient and the presence of the targeted tumor cells can be determined.

The following examples are offered solely for the purposes of illustration, and are intended neither to limit nor to define the invention.

EXAMPLE 1

This example illustrates the conjugation of doxorubicin to a monoclonal antibody.

Doxorubicin conjugates were prepared by a modification of the glutaraldehyde method (Acton et al., J. Med. Chem. 27:638-645 (1984), which is incorporated by reference herein). Briefly, a monoclonal antibody (mAb) directed against human EGF receptor, mAb 425 (Murthy et al., Arch. Biochem. Biophys. 252:549-560 (1987)) was purified from hybridoma supernatant fluids by affinity chromatography on protein-A sepharose (Repligen, Cambridge, MA). A negative control monoclonal antibody directed against a 40 kDa glycoprotein expressed on most epithelial tumors, monoclonal antibody NRLU-10 (Breitz et al., Nuclear Med. 33: 1099-1112 (1992)), was obtained from NeoRx Corporation (Seattle, WA). These murine monoclonal antibodies, 425 and NRU-10, were of IgG2a and IgG2b isotype, respectively. The antibodies were prepared in phosphate buffered saline (PBS), pH 7.2. The antibody (3-5 mg/mL) was reduced with dithiothreitol (DTT) at a final concentration of 25 mM for thirty minutes at room temperature. After reduction the reaction mixture was passed through a PD-10 column (Pharmacia, Piscataway, NJ) to remove unreacted DTT.

To prepare the doxorubicin intermediate, the amount of doxorubicin to be conjugated was determined by calculating a 25:1 molar ratio of doxorubicin (Sigma, St. Louis, MO) to antibody. Freshly distilled, concentrated glutaraldehyde (Polysciences) in a sealed vial was first opened and aliquoted into small volumes, sealed and frozen at -20°C until use. Immediately before use, the glutaraldehyde was thawed and diluted to the correct concentration with distilled water. An equal molar amount (1:1 molar ratio of doxorubicin:glutaraldehyde) of glutaraldehyde was added to the doxorubicin at room temperature. The doxorubicin-glutaraldehyde mixture was added to the reduced antibody, and the reaction mixture was mixed continuously on a rotary shaker for 1 hour at room temperature. Unreacted doxorubicin was

removed by PD-10 gel filtration, and the purity of the conjugate was assessed by gel filtration chromatography. The ratio of doxorubicin to antibody was calculated by measuring absorbance at 280 nm and 495 nm respectively and by correcting for the absorbance of doxorubicin at 280 nm. The antibody conjugate was stored at 4°C until use in the studies of Examples 2-5.

EXAMPLE 2

This example illustrates the binding of a doxorubicin conjugate to a human melanoma cell line.

The human melanoma cells, M24met (described previously in Mueller *et al.*, *Cancer Res.*, 51:2193-2198 (1991)), were grown in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. The cells were detached from tissue culture plastic with 0.5 mM EDTA, 0.15 M NaCl, 0.02 M 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, washed once, and used as a single cell suspension. The M24met cells were maintained in RPMI 1640 tissue culture medium (GIBCO BRL, Gaithersburg, MD) that was supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (Sigma Chemical Company, St. Louis, MO) and were tested monthly for mycoplasma contamination using the GenProbe kit (GenProbe, San Diego, CA) and found to be mycoplasma free. For the assay, the cells were grown to confluence in standard T flasks, detached, washed once in medium or PBS containing 1% FBS and 0.02% NaN₃, and used as a single cell suspension.

The amount of binding of monoclonal antibody-doxorubicin conjugates to M24met cells was determined by indirect immunofluorescence. Approximately 1×10^6 M24met cells were incubated for one hour at 4°C with either unconjugated monoclonal antibody or the doxorubicin-conjugated antibody. Unbound antibody or conjugate was removed by washing the cells with PBS containing 1% FBS and 0.02% NaN₃. The cells were then stained with goat anti-mouse IgG

antibodies conjugated to fluorescein isothiocyanate (Boehringer, Indianapolis, IN) for 1 hour at 4°C. Cells were analyzed in a FACSCAN flow cytometer (Becton Dickinson, Mountain View, CA). Indirect immunofluorescence analysis indicated specific binding of anti-EGFR monoclonal antibody 425 and of 425-DOX conjugates to M24met human melanoma cells *in vitro*. The conjugation procedure decreased binding of 425-doxorubicin by approximately 10-15% as compared to unconjugated monoclonal antibody 425 at equimolar concentrations. As a control conjugate NRU-10 antibody was conjugated with doxorubicin and incubated with the M24met cells. NRU-10-doxorubicin did not bind to M24met cells. Despite the 10-15% reduction in binding, M24met proliferation was inhibited in a dose dependent fashion by both doxorubicin and 425-doxorubicin conjugates. The 425-doxorubicin conjugate was approximately three-fold more efficient than an equimolar amount of free doxorubicin in the inhibition of M24met proliferation.

EXAMPLE 3

This example illustrates the *in vitro* cytotoxicity of monoclonal antibody-doxorubicin conjugates.

The cytotoxicity of monoclonal antibody-doxorubicin conjugates *in vitro* was determined by a [³H]-thymidine incorporation assay. To prepare cells for the cell proliferation assays, confluent M24met cells were detached by trypsinization or using the method described above. The cells were washed once with fresh medium and resuspended in fresh medium. Approximately 5×10^3 M24met cells were plated into each well of 96-well microtiter plates. Free doxorubicin or monoclonal antibody-doxorubicin conjugate was added to the appropriate wells, and the plates were incubated for 72 hours at 37°C. After 64 hours of incubation, the cells were labeled by adding 1 microcurie of [³H]-thymidine (Amersham, Arlington Heights, IL) to each well. Cells were harvested onto glass fiber filters using a PHD cell harvester (Cambridge

Technologies Inc., MA), and the incorporated radioactivity was determined by liquid scintillation counting. [³H]-Thymidine incorporation was determined and expressed as percentage of untreated controls in order to quantify cell proliferation.

5 Anti-EGFR monoclonal antibody 425 had no effect on the [³H]-thymidine incorporation of M24met cells (Fig. 2). The *in vitro* cytotoxicity of doxorubicin conjugates in 72 hr [³H]-thymidine incorporation assays was not antigen-specific under the conditions used for this experiment.

10

EXAMPLE 4

This example illustrates the activity of monoclonal antibody-doxorubicin conjugates in a subcutaneous tumor model.

A subcutaneous tumor model in *scid* mice was used to measure tumor growth in the presence and absence of antibody-conjugated doxorubicin. Six to eight-week old female C.B-17 *scid/scid* mice obtained from Scripps Research Institute (La Jolla, CA) were used in all experiments. Mice were housed under specific pathogen-free conditions and were handled in a laminar flow air cabinet.

20 Approximately 2×10^6 M24met cells in 100 μ L of culture medium were injected subcutaneously into the flank of *scid* mice. The animals were grouped for intravenous injection of either monoclonal antibody 425 (0.5 mg per dose), doxorubicin (15 μ g per dose), monoclonal antibody 425-doxorubicin conjugate (0.5 mg monoclonal antibody containing 15 μ g doxorubicin per dose), or PBS on days 2 and 4 after tumor inoculation. On day 2, tumor nodules were palpable. Tumors were measured with calipers and tumor volumes were estimated as $(a^2 \times b)/2$ where "a" represents the tumor width and "b" the tumor length. Mice were euthanized when tumor volumes exceeded 2000 mm^3 .

30 35 The 425-doxorubicin conjugate completely inhibited the growth of subcutaneous M24met tumors in *scid* mice. Fig. 3 illustrates the antitumor effect of this conjugate and the controls when injected at an equivalent dose of 0.5 mg

antibody and 15 µg doxorubicin 2 days and 4 days after tumor cell inoculation. Small tumor deposits measuring 50 mm³ persisted on day 7 after inoculation in mice treated with 425-doxorubicin, but these small tumors slowly disappeared,
5 leaving palpable lesions that remained for approximately 50 days. This group of mice was followed for 150 days, and during this time, all animals remained tumor-free. In contrast, the equivalent dose of free doxorubicin had no observable effect on tumor growth. Doxorubicin-treated mice
10 and untreated control mice were sacrificed 24 days after tumor cell inoculation with subcutaneous tumors weighing about 3 grams. Although an equivalent dose of monoclonal antibody 425 reduced the growth rate of subcutaneous M24 met tumors initially, all mice had very large tumors on day 39 and were
15 euthanized.

EXAMPLE 5

This example illustrates the activity of monoclonal antibody-doxorubicin conjugates in a spontaneous metastasis model.

20 M24met cells (2×10^6 in 100 µL) were injected subcutaneously into the flank of *scid* mice to establish a model system for spontaneous metastasis. When the subcutaneous tumors reached a volume of 1000 mm³, usually 15 days after injection, the mice were anesthetized with an
25 intraperitoneal injection of 2.5 mg ketamine, and the subcutaneous tumors were excised under aseptic conditions. The mice were separated into groups and were injected intravenously with either monoclonal antibody 425 (0.25 mg per dose), doxorubicin (7.5 µg per dose), monoclonal antibody 425-doxorubicin conjugate (0.25 mg monoclonal antibody containing
30 7.5 µg doxorubicin per dose), NRU-10-doxorubicin conjugate (0.25 mg monoclonal antibody containing 7.5 µg doxorubicin per dose), or PBS at one and four days after excision of the primary tumor. This optimum dose was selected based on a dose response study using monoclonal antibody 425-doxorubicin
35

conjugate in the same model. The extent of metastasis in the treatment groups was evaluated 21 days after removal of the primary tumor. Lungs were fixed in Bouins solution and tumor foci on the surface were counted under low magnification.

5 Enlarged lymph nodes were excised, weighed, and fixed in 10% buffered formalin. Histological sections from representative lymph nodes were stained with hematoxylin and eosin. Metastases to ipsilateral lymph nodes and lungs were found in 100% of these mice. Histological analyses of representative 10 lymph node sections showed replacement by malignant polygonal melanoma cells. Obvious metastatic foci were visible on the surface of the lungs, and histological analysis indicated numerous nests and nodules of metastatic tumors in the alveolar spaces.

15 Activity and specificity of 425-doxorubicin were determined by comparing it to treatments with either monoclonal antibody 425 alone, free doxorubicin or a nonbinding control conjugate, NRLU-10-doxorubicin. At 21 days after excision of the primary tumors, all mice injected 20 with either saline, monoclonal antibody 425 or doxorubicin had massive metastasis to the draining lymph nodes and lungs. In contrast, half of the mice treated with the 425-doxorubicin conjugate showed no evidence of metastasis in lungs or lymph nodes. Whenever metastasis did occur in lymph nodes and lungs 25 of these animals, they were reduced in size or number or both when compared to control groups treated with either saline or doxorubicin. Treatment with monoclonal antibody 425 alone also reduced the number of lung metastases. In this group, however, more than 80% of the mice showed evidence of lung 30 metastases as compared to less than 20% observed in the group treated with 425-doxorubicin. The control conjugate NRLU-10-doxorubicin also suppressed the growth of melanoma metastases. While its antitumor effect was not as pronounced as that of 425-doxorubicin, it was still better than that of free 35 doxorubicin. Treatment with antibody-doxorubicin conjugates at the described dose levels and schedule caused no change in body weight, suggesting the absence of overt toxicity.

For survival experiments, groups of mice were treated as described above, except, instead of sacrificing the mice 21 days after excision of the primary tumor, the animals were observed and sacrificed when tumors started to cause obvious distress.

Monoclonal antibody 425-doxorubicin conjugates were found to prolong the survival of *scid* mice with human melanoma metastasis. This was evident from experiments determining the life span of mice after treatment with either 425-doxorubicin, 10 NRLU10-doxorubicin or doxorubicin following surgical excision of the primary M24met tumors. All mice which had been treated with a total dose of 15 µg free doxorubicin died with extensive metastasis within 30 to 45 days (Fig. 5). Groups of animals which had been treated with equivalent doses of either 15 425-doxorubicin or NRLU-10-doxorubicin showed improved survival. Thus, 150 days after excision of the primary tumors, 50% of mice treated with 425-doxorubicin survived without signs of metastatic disease compared to 25% survivors in the group treated with NRLU-10-doxorubicin. Among the 20 three agents tested at a single molar equivalent dose of doxorubicin, the specific monoclonal antibody 425-doxorubicin conjugate proved to be the most effective, whereas free doxorubicin was least effective in suppressing dissemination and growth of melanoma metastases in *scid* mice.

25

EXAMPLE 6

This example describes a comparison of results achieved with the doxorubicin conjugate of the present invention with those of a doxorubicin-conjugated antibody prepared using standard conjugation via a hydrazide intermediate. Doxorubicin conjugates were prepared using the method set forth by Kaneko et al. (U.S. Patent No 5,137,877; which is incorporated by reference herein in its entirety) and Willner et al. (Bioconjugate Chem. 4: 521-527 (1993); which is incorporated by reference herein in its entirety) using mAB 425 (Example 1). Conjugates prepared according to the method of Kaneko et al. (*ibid.*) were referred to as 425/dox(hyd).

Doxorubicin conjugates prepared according to the present invention (Example 1) were referred to as 425/dox(glu). In addition, the doxorubicin hydrazide intermediate from the Kaneko et al. (*ibid.*) method was isolated and compared in cytotoxicity assays with the two conjugates.

In a similar manner, pairs of doxorubicin conjugates using the two methods were also prepared using the NRU10 antibody. Conjugates prepared according to the method of Kaneko et al. (*ibid.*) were referred to as NRU10/dox(hyd), and doxorubicin conjugates prepared according to the present invention (Example 1) were referred to as NRU10/dox(glu).

The cytotoxicity of 425-doxorubicin conjugates was determined by an *in vitro* [³H]-thymidine incorporation assay as described in Example 3. Briefly, confluent M24met cells were detached and the cells were washed once with fresh medium and resuspended in fresh medium. Approximately five thousand M24met cells were plated into each well of 96-well microtiter plates. Free doxorubicin, 425/dox(glu) conjugate, the 425/dox(hyd) conjugate or the doxorubicin hydrazide intermediate at increasing concentrations were added to the appropriate wells (2500 ng, 250 ng, 25 ng and 2.5 ng doxorubicin equivalents). The plates were incubated for 72 hours at 37°C. After 64 hours of incubation, the cells were labeled by adding 1 millicurie of [³H]-thymidine (Amersham) to each well. Cells were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technologies Inc.), and the incorporated radioactivity was determined by liquid scintillation counting. [³H]-thymidine incorporation was determined and expressed as percentage of untreated controls in order to quantify cell proliferation. As shown in Table 1, the 425/dox(glu) conjugate of the present invention was more cytotoxic than the 425/dox(hyd), free doxorubicin or the doxorubicin hydrazide intermediate.

Table 1

Doxorubicin concentration	425/dox (glu)	425/dox (hyd)	Free doxorubicin	Doxorubicin hydrazide
2500 ng	0.65	29.4	9.8	4.3
250 ng	5.4	81.6	8.4	36
25 ng	36.4	68.3	23.6	56.2
2.5 ng	55.9	69.2	57.6	82.3

5 Doxorubicin resistance is a significant problem in chemotherapy regimes. Thus, the efficacy of the conjugates of
10 the present invention was tested against doxorubicin resistant MCF7 cells. Cytotoxicity assays were carried out essentially as described above using NRLU10/dox(glu), NRLU10/dox(hyd) and free doxorubicin. The results of the assay, shown in Table 2, demonstrate that the NRLU10/dox(glu) was more cytotoxic than
15 either NRLU10/dox(hyd) or free doxorubicin.

Doxorubicin concentration	NRLU10/dox (glu)	NRLU10/dox (hyd)	Free doxorubicin
2500 ng	18.6	95.2	70.9
250 ng	56.2	110.2	89.5
25 ng	72	105.2	103.4
2.5 ng	91	110.7	113.9

20 All publications and patents mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated herein by reference.

25 The above description is illustrative and not restrictive. Many variations of the invention will become apparent to those of skill in the art upon review of this disclosure. Merely by way of example a variety of compounds, dialdehyde, antibodies, conditions for reaction and protocols for treatment may be used without departing from the scope of the invention. The scope of the invention should, therefore,
30 be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

WHAT IS CLAIMED IS:

1. A targeting molecule-effector moiety conjugate having the formula:

5



wherein,

D^1-N represents a condensation product of an effector moiety having a primary amine group;

10 $Y^1-P^1-Y^2$ represents a targeting molecule having side-chain functionality Y^1 and Y^2 which are independently selected from the group consisting of $-SH$ and $-NH_2$;

X is a member selected from the group consisting of $-O-$, $-S-$, $-CH_2-$, and $-CH(R)-$; and

15 R , R^1 and R^2 are each members independently selected from the group consisting of H, lower alkyl, lower alkoxy, halogen, lower alkylthio, and lower acyloxy.

20 2. A conjugate in accordance with claim 1 wherein D^1-N represents a condensation product of a drug and X is a member selected from the group consisting of $-O-$, $-S-$, $-CH_2-$, and $-CH(R)-$.

25 3. A conjugate in accordance with claim 2 wherein the drug is an antineoplastic agent.

25

4. A conjugate in accordance with claim 3 wherein the antineoplastic agent is selected from doxorubicin, bleomycin, vinblastine, vincristine, etoposide and etoposide phosphate, paclitaxel, teniposide, daunomycin, melphalan, cyclophosphamide, chlorambucil, cisplatin, carboplatin, methotrexate, podophyllotoxin, verrucarin A, anguidine, mechlorethamine, leucovorin, cytosine or adenine arabinosides, 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodocytosine, 5-

iodo-5'-amino-2',5-dideoxyuridine, 5'-amino-5'-deoxythymidine, mitomycin, 1-amantanamine, rimantadine, and tamoxifen.

5 5. A conjugate in accordance with claim 3 wherein D¹-N represents a condensation product of doxorubicin and X is -CH(OCH₃)-.

10 6. A conjugate in accordance with claim 1 wherein D¹-N represents a condensation product of daunomycin and X is -CH₂-.

15 7. A conjugate in accordance with claim 1 wherein D¹-N represents a condensation product of doxorubicin, Y¹-P¹-Y² represents a reduced monoclonal antibody and X is -CH₂- or -O-.

20 8. A conjugate in accordance with claim 1, wherein the targeting molecule is a protein, carbohydrate or oligonucleotide.

25 9. A conjugate according to claim 8, wherein the protein is a monoclonal antibody or an antigen binding fragment thereof.

10. A conjugate according to claim 9, wherein the monoclonal antibody or binding fragment binds to a tumor cell associated antigen.

30 11. A targeting molecule-effector moiety conjugate having the formula:



wherein,

D¹-NH represents a condensation product of an effector moiety having an amino group;

$\text{Y}^3\text{-P}^1$ represents a targeting molecule having a side-chain functionality Y^3 which is $-\text{SH}$ or $-\text{NH}_2$; and

R^3 is a member selected from the group consisting of an alkyl group, an alkoxy group, an alkylthio group, a halogen, a carboxylic ester group and a carboxylic acid group.

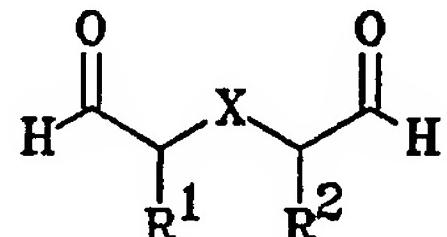
12. A conjugate in accordance with claim 11 wherein $\text{D}^1\text{-N}$ represents a condensation product of an antineoplastic agent and X is a member selected from the group consisting of $-\text{O}-$, $-\text{S}-$, $-\text{CH}_2-$, and $-\text{CH}(\text{R})-$.

13. A conjugate in accordance with claim wherein $\text{D}^1\text{-N}$ represents a condensation product of doxorubicin and X is $-\text{CH}(\text{OCH}_3)-$.

14. A conjugate in accordance with claim 11 wherein $\text{D}^1\text{-N}$ represents a condensation product of daunomycin and X is $-\text{CH}_2-$.

15. A method of preparing a targeting molecule-effector moiety conjugate comprising,

(a) treating an effector moiety having an attached primary amine group with a dialdehyde having the formula:



25. wherein,

X is a member selected from the group consisting of $-\text{O}-$, $-\text{S}-$, $-\text{CH}_2-$, and $-\text{CH}(\text{R})-$; and

30. R, R^1 and R^2 are each members independently selected from the group consisting of H, lower alkyl, lower alkoxy, halogen, lower alkylthio and lower acyloxy, to produce a derivatized compound; and

(b) contacting a targeting molecule having at least two side chain functionalities selected from the group consisting of $-\text{SH}$ and $-\text{NH}_2$ with said derivatized compound

under conditions sufficient to covalently attach said targeting molecule to said derivatized compound at two sites to produce said targeting molecule-effector moiety conjugate.

5 16. A method in accordance with claim 15 wherein said targeting molecule is a protein.

10 17. A method in accordance with claim 16, wherein said protein is a reduced monoclonal antibody.

15 18. A method in accordance with claim 15 wherein said effector moiety is a therapeutic agent selected from the group consisting of doxorubicin, bleomycin, vinblastine, vincristine, etoposide and etoposide phosphate, paclitaxel, teniposide, daunomycin, melphalan, cyclophosphamide, chlorambucil, cisplatin, carboplatin, methotrexate, podophyllotoxin, verrucarin A, anguidine, mechlorethamine, leucovorin, cytosine or adenine arabinosides, 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodocytosine, 5-iodo-5'-amino-2',5-dideoxyuridine, 5'-amino-5'-deoxythymidine, mitomycin, 1-amantanamine, rimantadine, and tamoxifen.

20 19. A method in accordance with claim 15 wherein said effector moiety is doxorubicin.

25 20. A method in accordance with claim 15 wherein said effector moiety is doxorubicin and said dialdehyde is glutaraldehyde.

30 21. A conjugate prepared according to the method of claim 15.

35 22. A method of preparing a targeting molecule-effector moiety conjugate comprising,

(a) treating an effector moiety having an attached primary amine group with a monoaldehyde to produce a derivatized compound; and

(b) contacting a targeting molecule having a side chain functionality selected from the group consisting of -SH and -NH₂ with said derivatized compound under conditions sufficient to produce said conjugate.

5

23. A method in accordance with claim 22, wherein said effector moiety is a therapeutic agent and said targeting molecule is a protein, carbohydrate or oligonucleotide.

10

24. A method for treating a patient suffering from a tumor or other tissue malignancy, said method comprising administering to said patient a therapeutically effective amount of a targeting molecule-effector moiety conjugate selected from the group consisting of conjugates of formula:

15



wherein,

D¹-N represents a condensation product of an effector moiety having a primary amine group;

20 Y¹-P¹-Y² represents a targeting molecule having side-chain functionality Y¹ and Y² which are independently selected from the group consisting of -SH and -NH₂;

 X is a member selected from the group consisting of -O-, -S-, -CH₂-, and -CH(R)-; and

25 R, R¹ and R² are each members independently selected from the group consisting of H, lower alkyl, lower alkoxy, halogen, lower alkylthio and lower acyloxy;
and conjugates of formula II:



wherein,

D¹-NH represents a condensation product of an effector moiety having a free amino group;

5 y³-P¹ represents a targeting molecule having a side-chain functionality y³ which is -SH or -NH₂; and

R³ is a member selected from the group consisting of an alkyl group, an alkoxy group, an alkylthio group, a halogen, a carboxylic ester group and a carboxylic acid group.

10 25. A method in accordance with claim 24 wherein said targeting molecule-effector moiety conjugate is a conjugate of formula I.

15 26. A method for treating a patient suffering from a tumor or other tissue malignancy, said method comprising administering to said patient a therapeutically effective amount of a targeting molecule-effector moiety conjugate of claim 21.

20 27. A pharmaceutical composition comprising at least one targeting molecule-effector moiety conjugate selected from the group consisting of conjugates of formula I:



wherein,

25 D¹-N represents a condensation product of an effector moiety having a primary amine group;

y¹-P¹-y² represents a targeting molecule having side-chain functionality y¹ and y² which are independently selected from the group consisting of -SH and -NH₂;

30 X is a member selected from the group consisting of -O-, -S-, -CH₂-, and -CH(R)-; and

R, R¹ and R² are each members independently selected from the group consisting of H, lower alkyl, lower alkoxy, halogen, lower alkylthio and lower acyloxy; and conjugates of formula II:

5



wherein,

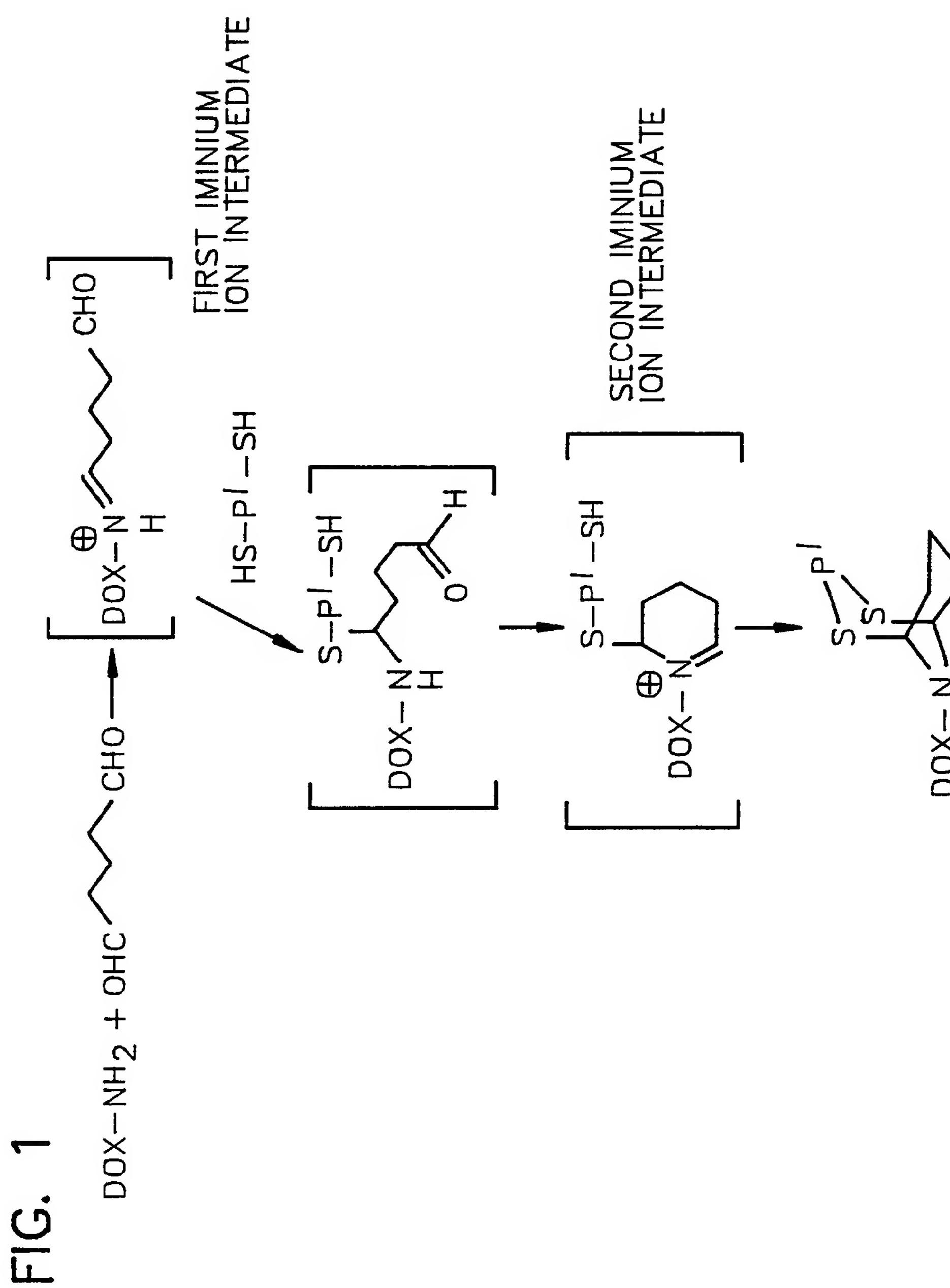
D¹-NH represents a condensation product of an effector moiety having a free amino group;

10 Y³-P¹ represents a targeting molecule having a side-chain functionality Y³ which is -SH or -NH₂; and

R³ is a member selected from the group consisting of an alkyl group, an alkoxy group, an alkylthio group, a halogen, a carboxylic ester group and a carboxylic acid group; and a pharmaceutically acceptable carrier.

15

1/2



2/2

FIG. 2

INHIBITION OF
 ^3H -THYMIDINE INCORP.
[% OF UNTREATED
CONTROL]

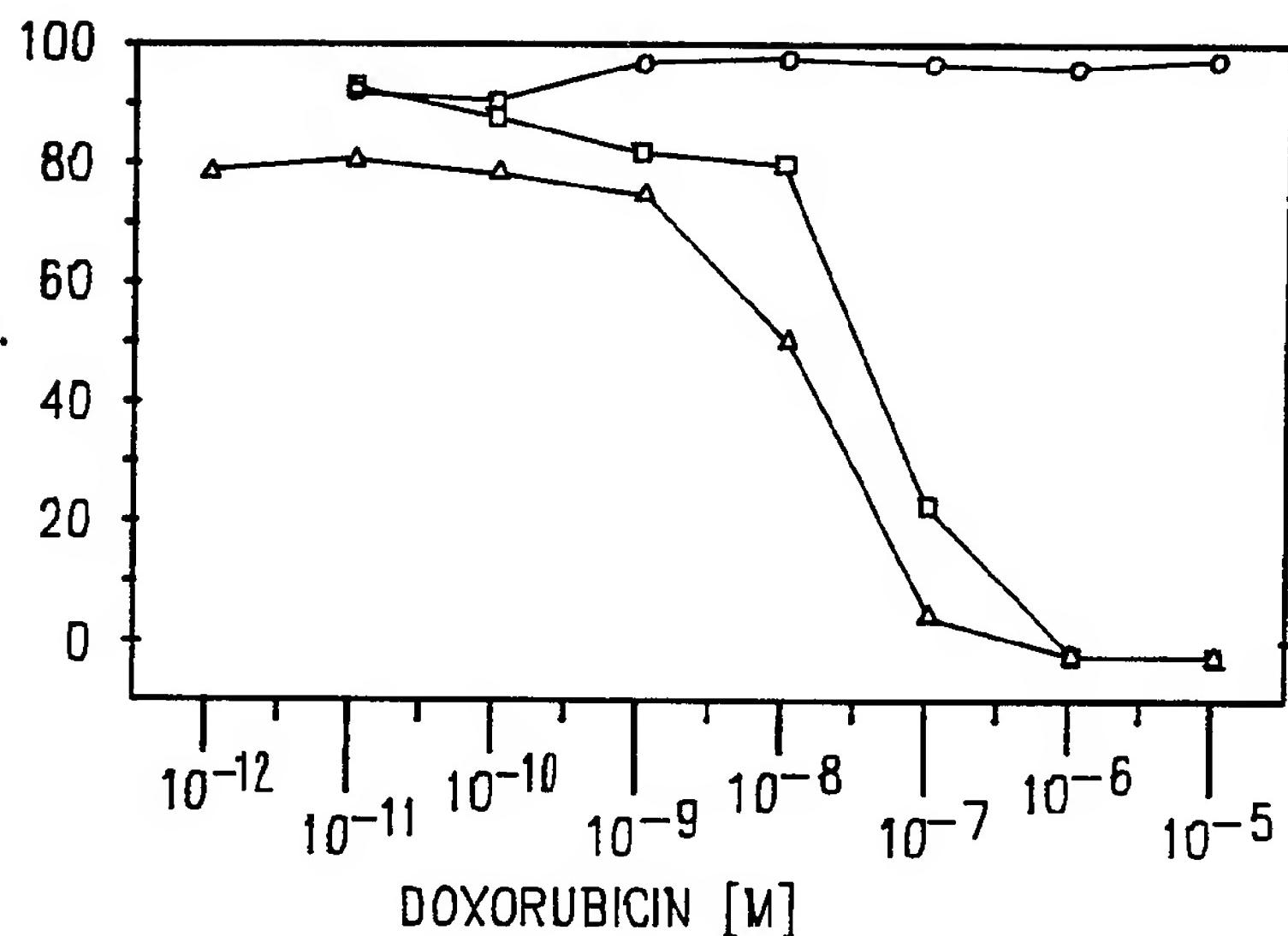


FIG. 3

TUMOR
VOLUME
[mm³]

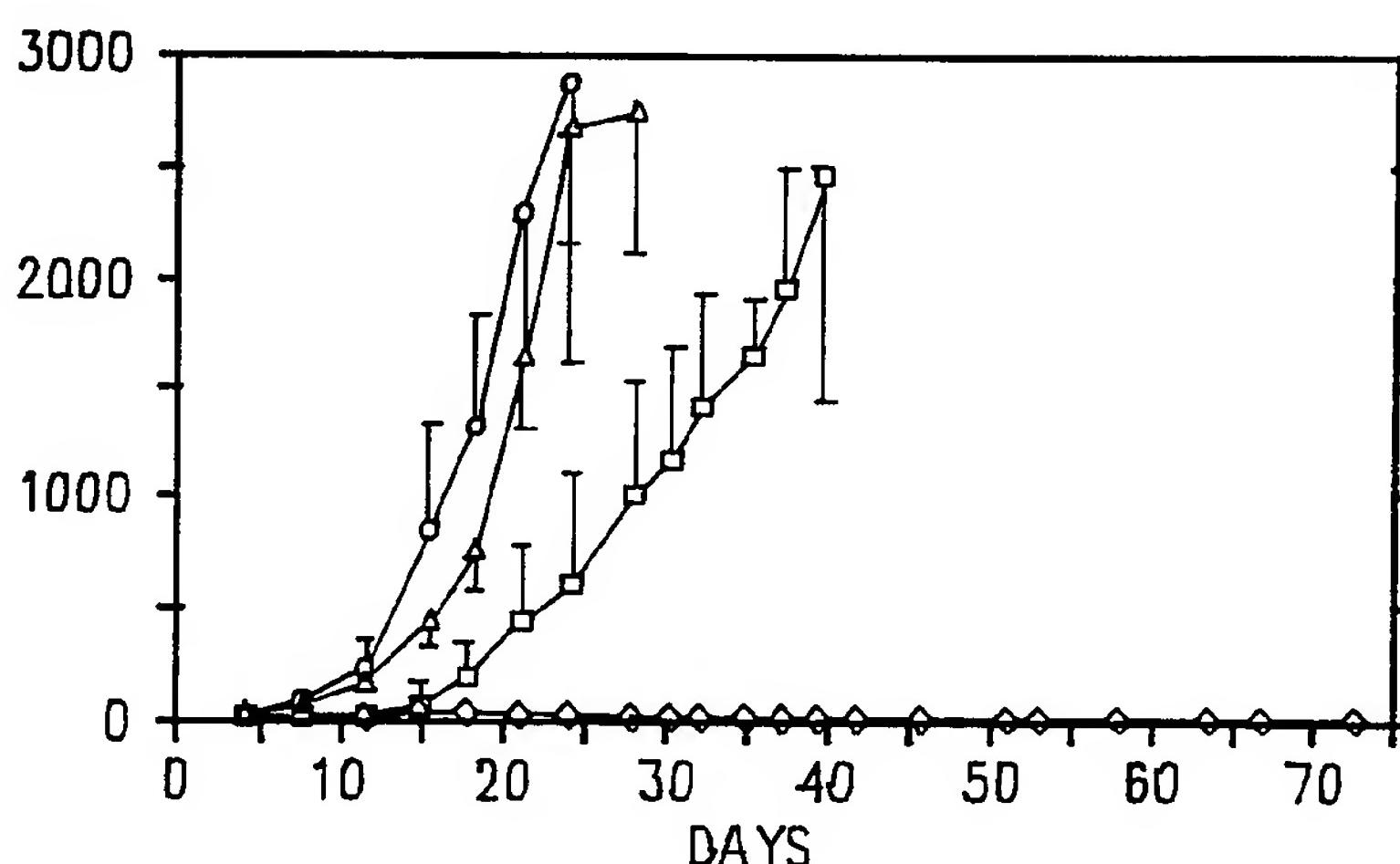
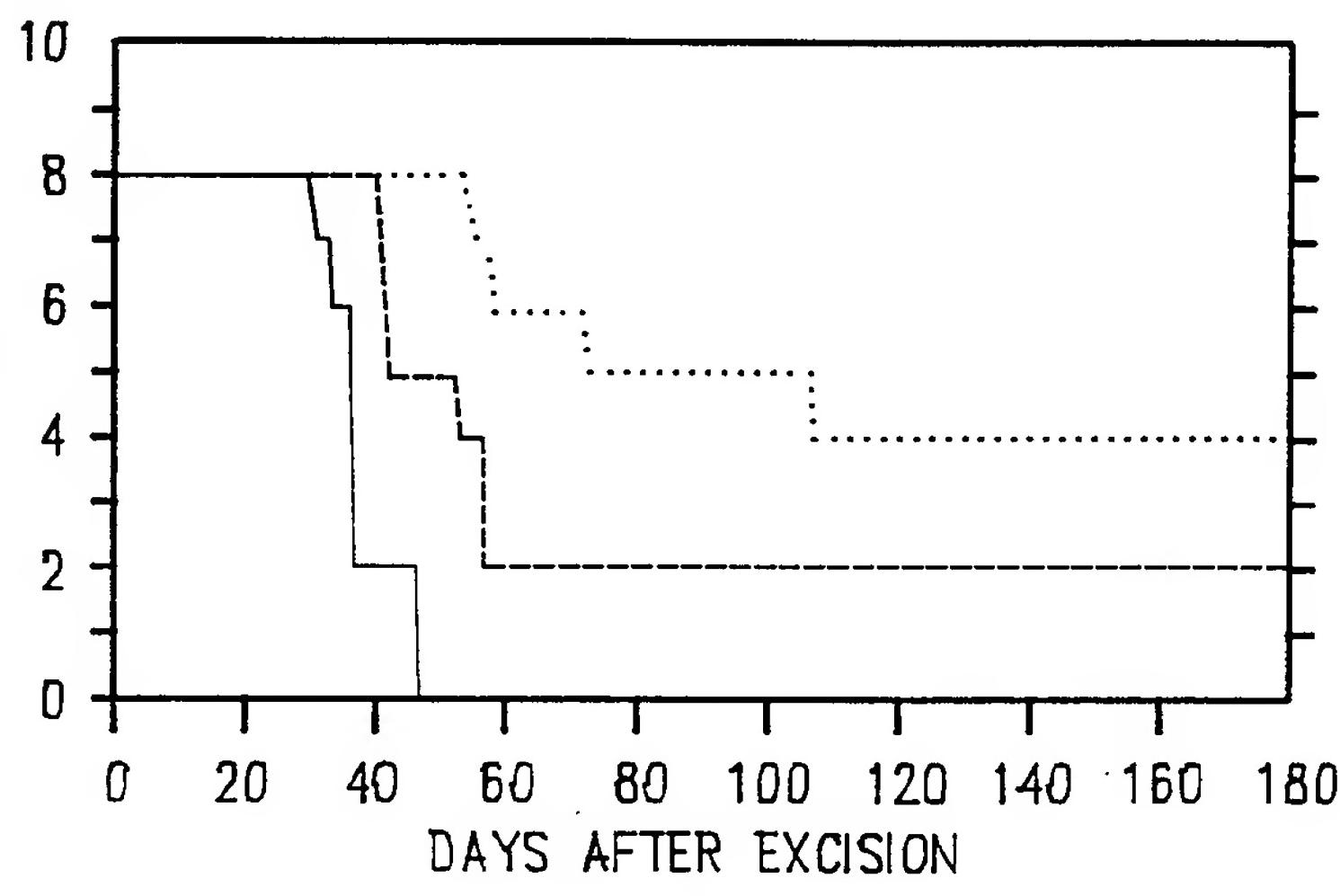


FIG. 4

SURVIVING
MICE



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB96/00588

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/395; C07K 16/00

US CL : 424/181.1, 179.1, 178.1; 530 391.9, 391.7, 391.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/181.1, 179.1, 178.1; 530 391.9, 391.7, 391.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,116,944 (SIVAM et al.) 26 May 1992, columns 8 and 9.	1-27
Y	US, A, 5,208,323 (PAGE et al.) 04 May 1993, column 2, lines 45-61; column 3 to column 6 and column 8, claims 1-6.	1-27
Y	US, A, 5,162,218 (SCHULTZ) 10 November 1992, columns 7-9.	1-27
A	US, A, 5,135,736 (ANDERSON et al.) 04 August 1992, column 1, lines 16-34.	1-27
A	US, A, 5,420,105 (GUSTAVSON et al.) 30 May 1995, column 2, line 27.	1-27

 Further documents are listed in the continuation of Box C.


See patent family annex.

* Special categories of cited documents:	"T"	Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
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P document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CA-A Cancer Journal for Clinicians, Vol. 44, No. 1, issued January/February 1994, Goldenberg ", New Developments in Monoclonal Antibodies for Cancer Detection and Therapy, pages 43-64, entire document.	1-27
Y, P	Cancer Research, Volume 55, Number 11, issued 01 June 1995, Sivam et al, "Therapeutic Efficacy of a Doxorubicin Immunoconjugate in a Preclinical Model of Spontaneous Metastatic Human Melanoma", page 2352, column 2 to page 2353.	1-27
Y	Molecular Immunology, Volume 27, Number 3, issued 1990, Morgan, Jr., "Immunotoxins of Pseudomonas Exotoxin a (PE): Effect of Linkage on Conjugate Yield, Potency, Selectivity and Toxicity", pages 273-282, see page 274, columns 1 and 2.	1-27